

Antibacterial Evaluation and Phytochemical Analysis of Selected Medicinal Plants against Some Pathogenic Enteric Bacteria in Gozamin District, Ethiopia

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Abstract

Antibacterial activity evaluation and Phytochemical Analysis of aqueous, chloroform, methanol and ethanol crude extracts of thirteen medicinal plants species that were selected based on ethno botanical information on their traditional use were tested for treatment of enteric disease in Gozamin District. The study has been carried out from January 5, 2014 to February 15, 2015. All of these plants were extracted following standard methods (Soaking extraction method and agar-well diffusion) to screen of potential anti-microbial substance. All crude extracts of those medicinal plants were tested against standard reference strains including *Escherichia coli*, *Staphylococcus aureus*, *Shigella sonnei*, *Pseudomonas aeruginosa* and *Salmonella typhimurium*. The highest antibacterial activity (17 mm) was observed from chloroform leaf extract of *Eucalyptus globules* against *E. coli* and leaf extract of *Verbena officinalis* (13.6 mm) extract against *Shigella sonnei*, followed by methanol leaf extract of *Cordia africana* (12.8 mm) against *E. coli*. More over *Eucalyptus globules* was positive for all bioactive compounds tested except saponins and *Verbena officinalis* was positive for all bioactive ingredients tested except alkaloids. In general extracts of *Eucalyptus globules* leaves exhibited the highest potency against *E. coli* and extract *Verbena officinalis* showed highest potency against *Shigella sonnei*. Thus, this study confirmed the alternative sources of medicine for pathogenic enteric bacteria tested.

Keywords: Enteric disease; Crude extracts; Phytochemical studies

Introduction

Plants have not only nutritional value but also, in the eyes of the local people, they have medicinal and ritual or magical values [1]. Traditional medicinal plants have important contributions in the health care system of local communities as the main source of medicine for the majority of the rural population. These medical systems are heavily dependent on various plant species and plant based products. Since time immemorial, plants have been indispensable sources of both preventive and curative traditional medicine preparations for human beings and livestock [2]. There are more than 35,000 plant species being used in various human cultures around the world for medicinal purpose [3].

According to WHO, over 80% of the world's population relies upon traditional plant-based systems of medicines to provide them with primary healthcare [4]. Fransworth and Soejarto [5] also echoed the same with their estimation that 70-80% of people worldwide rely chiefly on traditional, largely herbal medicine to meet their primary healthcare needs. It is further indicated that herbal medicine is still the mainstay of about 75-80% of the world population, mainly in the developing countries, for primary healthcare. This is primarily because of the general belief that herbal drugs are without any side effects besides being cheap and locally available [6].

Researchers are still searching for the potential antimicrobial substances from medicinal plants due to the increasing failure of chemotherapeutics and antibiotic resistance exhibited by pathogenic microorganisms [7]. It is important to note that microbial diseases continued to be major threats to the world regardless of efforts and progress in developing modern medicine [8]. The impact of microbial diseases is especially important in developing countries such as Ethiopia where there is limited access to modern drugs and prices are mostly unaffordable when the latter are available [9]. A focused phytochemical

screening, backed by ethnomedicinal data, often leads to the discovery of new lead compounds that can play a role in the global efforts against pathogens [10].

The emergence and spread of multidrug-resistant (MDR) enteric bacterial pathogens have substantially threatened the current antibacterial therapy [11]. MDR enteric bacterial infections often lead to increased mortality, longer length of stays in hospitals, and higher cost of treatment and care [11] and [12]. The therapeutic alternatives for these pathogens are extremely limited and physicians are forced to use expensive or previously discarded drugs, such as colistin, that are associated with significant side effect to the patients' health [12]. Therefore, it is necessary to search other potential alternatives that can be effective in the treatment of these problematic bacterial infections. In addition to this problem, antibiotics are sometimes associated with adverse effects on the host including hypersensitivity, immune-suppression and allergic reactions. This situation forced scientists to search for new antimicrobial substances.

In Ethiopia, medicinal plants have been used as traditional medicine to treat different human and livestock ailments by the local people from time immemorial. Ethiopian plants have shown very effective

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medicinal value for the treatment of virus diseases. Medicinal plants and knowledge of their use provide a vital contribution to human and livestock healthcare in the country at large [13]. Due to its long period of practice and existence, traditional medicine has become an integral part of the culture of Ethiopian people. There is a large magnitude of use and interest in medicinal plants in Ethiopia due to acceptability, accessibility and biomedical benefits [14]. The same is true to people living in gozamen district and hence they are highly dependent on medicinal plants for their primary healthcare.

Antimicrobial evaluation of studies is often significant in revealing locally available and important plant species especially for the discovery of crude drugs. In Ethiopia, has a long history of traditional healthcare system, however studies conducted on the traditional medicinal plants are limited when compared with the multiethnic, cultural and flora diversity [15]. Hence this study would contribute to fill the existing gap in determining the antimicrobial effect of selected traditional plants and to declare bioactive compounds found in each evaluated medicinal plants in Gozamin District.

Therefore the study was necessitated to evaluate the antimicrobial effect of some commonly used potential medicinal plants and its phytochemical test in Gozamen District. This could attribute to the effective utilization of natural resources in the locality there by transforming the traditional knowledge to scientific way of utilization of those medicinal plants to be used as raw material to pharmaceutical industries, as well as to conserve rare or highly demanded plant species in traditional medicine.

Objectives of the Study

General objective

The main objective of this research was evaluation on antibacterial activity of some selected traditionally used medicinal plants against pathogenic enteric bacteria and determination of the bioactive compounds.

Specific objectives

- To evaluate the antibacterial activity of medicinal plants on enteric bacteria pathogens
- To identify the class of most common phytochemicals present in the selected medicinal plants
- To estimate the metabolic activity of the plant extract

Materials and Methods

Study area

Gozamin district is one of the 18 districts in east gojjam zone and 151 districts in amahara national regional state. The relative location of the district is 300 km away from the capital city of the country, addis ababa and 260 km from bahir dar, the regional capital city. This district is found almost mid-way from addis ababa to bahir dar. The district is bounded by senan district in the north, basoleben district and oromiya national regional state in the south, aneded and debaytilatgen district to the east and machakle and debere elias district in the west [16]. Medicinal plants were collected in kebeles of gozamin district. The antimicrobial evaluation was made in biology department microbiological laboratory and the phytochemical analysis was made in chemistry department analytical chemistry laboratory of debre markos university.

Plant materials

Plant specimens were collected from gozamin district between 25 June and 26 September 2009, 9 January-20 February 2010, 22 May-27 August 2010 and 14 February-7 May 2011.

Selection of medicinal plants

Thirteen medicinal plant species with relatively high informant consensus value for treating infectious diseases in the study area were selected for further antimicrobial activity study [17]. The thirteen plants were prioritized as common important for the treatment of gastro-intestinal disease in the study area. Local use reports of candidate medicinal plants from the study area were also thoroughly compared for related ethnomedicinal use reports from other parts of the country

These plants are (*E. globules*, *Rhamnus prinoides*, *Verbena officinalis*, *Otostigia integrifolia*, *Brucea antidysenterica*, *Sida schimperiana*, *Carica papaya*, *Phytolacca dodecandra*, *Cordia Africana*, *Vernonia amygdalina*, *Foeniculum vulgare*, *Withania somnifera*, *Cymbopogans martini*).

Extraction of plant material via maceration (Soaking)

Each plant samples were prepared following Panthi and Chaudhary, (2006) method of extraction; clean dry (dried under shade) plant samples were collected in cotton bags. The material was grinded using a grinder (blender). Each selected medicinal plant 40 g of sample was immersed in each of three conical flask containing 400 ml ethanol, 400 ml methanol and 400 ml chloroform solvent. The soaked samples were stayed for 72 hours with shaking of the extracts in the intermediate time. After 3 days the extract was filtered by using standard filter paper what man number 1. The collected extract was further separated by rotary evaporator at 40°C reduced temperature. Finally the crude extract was placed in desiccators containing CaCl_2 . The dried extract was put in refrigerator for further uses. The dry weight of the extracts were obtained by allowing the solvent to evaporate and also used to determine concentration in mg/ml.

Culture media used

Muller Hinton Agar (Oxoid, UK), Nutrient Broth (Himedia, India) and Nutrient Agar (Science Company, UK) were used during the study. Muller Hinton agar was used for the antimicrobial tests and Nutrient Agar was also used for routine stock cultures and sub culturing.

Test microorganisms and microbial culture

Four gram negative bacteria; (*Escherichia coli* ATCC 25722, *Salmonella typhimurium* ATCC 13311, *Shigella sonnei* ATCC259131 and *Pseudomonas aeruginosa* ATCC27853) and one Gram positive bacteria (*Staphylococcus aureus* ATCC 25903) known to cause food borne bacterial infection were used to evaluate the antimicrobial activity of crude extracts of medicinal plants. The test microorganisms were obtained from Ethiopian Health and Nutrition Research Institute (EHNRI) clinical bacteriology laboratory, addis ababa. The Bacterial strains were reactivated by sub culturing on nutrient broth at 37°C and maintained on nutrient agar slant at 4°C for further activity.

Standardization of inocula

The 0.5 McFarland turbidity standard was prepared by adding 0.5 ml of a 1.175% (wt/vol) barium chloride dehydrate ($\text{BaCl}_2 \cdot 2\text{H}_2\text{O}$) solution to 99.5 ml of 1% (vol/vol) sulfuric acid (H_2SO_4). This mix was considered to be equivalent to cell density of 1×10^8 cfu/ml [18]. The turbidity standard is then aliquoted into test tubes identical to

those used to prepare the inoculums suspension. McFarland turbidity standard tubes were Seal with Parafilm, to prevent evaporation. Barium sulfate turbidity was compared with identical tubes containing inocula 0.85% NaCl saline solution.

Agar disk diffusion assay

Agar disk diffusion method was used to evaluate the antibacterial activities of extracts of medicinal plants according to [19]. The 24 hours plate cultures of 0.5 Mc Farland standard (1 to 2×10^8 CFU ml⁻¹) bacterial suspensions were uniformly spread on Mueller-Hinton agar plate (Oxoid) to form lawn cultures. The chloroform, methanol and ethanol crude extracts were dissolved in tween -20 solvent. The stock solutions were prepared at amount of 100 mg/ml for each solvent extracts. The blotting paper discs (6 mm diameter) were soaked in various dilute solvent extracts, and dried for 5 minutes to avoid flow of extracts in the test media. Antibacterial activity of potential plant extract against bacterial pathogens by disc diffusion technique were identified after incubation for 24 h. at 37°C, and the result was obtained by measured the zone of inhibition of growth in mm. Standard antibiotic disc (Ampicilin 30 µg) were used as positive control.

Phyto-chemical analysis

Eight traditional plants which were found positive antimicrobial effect were further tested for the most common Phyto-chemicals (secondary metabolites) such as alkaloids, glycosides, flavonoids, tannins, saponins and phenolics present in powdered forms were analyzed following methods described in Trease and Evans [20], Ayoola GA et al. [21] Rasool R et al. [22].

Test for tannins

Half gram of the powdered plant materials were boiled in 10 ml of distilled water in a 100 ml beaker sized and then filtered; few drops of 0.1% ferric chloride (FeCl₃) were added. Formation of brownish green or a blue-black coloration indicates the presence of tannins [21].

Test for alkaloids

From about 0.5 g of powdered plant materials boiled in 10 ml of prepared acid alcohol and filtered, about 5 ml of the filtrate was taken and 2 ml of dilute ammonia added. Then 5 ml of chloroform was also added and shaken gently. The chloroform layer was extracted with 10 ml of acetic acid. Formation of a cream with Mayer's reagent confirms the presence of alkaloids [21].

Test for saponins

To 0.5 g of powdered plant materials in a test tube, 5 ml of distilled water was added and the mixture was vigorously shaken. Formation of a froth Persistent for 30 min confirms the presence of saponins [21].

Test for flavonoids

To a portion of an aqueous filtrate of the powdered plant materials about 5 ml of dilute ammonia solution was added. Concentrated sulphuric acid (1 ml) addition and yellow colorations that disappeared on standing indicated the presence of flavonoids [21].

Test for cardiac glycosides

To 2 ml alcoholic filtrate plant materials, 1 ml glacial acetic acid and 1-2 drops of FeCl₃ was added and 1 ml of concentrated H₂SO₄ followed. Appearance of brown ring at the interface indicated presence of cardiac glycosides. A violet ring also appeared below the brown ring confirm positive for cardiac glycosides [20].

Test for phenolics

To 2 ml of alcoholic or aqueous plant filtrate, 1 ml of 1% ferric chloride solution was added. After a minute appearance of blue or green color indicates presence of phenols [21].

Data analysis

Results obtained in this study were expressed as mean inhibition zone (mm) \pm S.D of three replicates. The mean and the standard deviation of each herbal extract were used to compute the calculated t-value.

The phytochemical tests were recorded as + (plus sign) for positive results for the tested bioactive compounds and - (minus sign) for the negative results for the tested result.

Results

Antibacterial activity of medicinal plants extracted by soaking method

The results showed that the among thirteen selected commonly used traditional medicinalplants only eight medicinal plant species, namely: *E. globules*, *Otostegia integrifolia*, *Verbena officinalis*, *Cordia Africana*, *Foeniculum vulgare*, *Withania somnifera*, *Phytolacca dodecandra* and *Brucea antidysenterica* were displayed positive effect on standard reference of enteric pathogenic bacterial strains *E. coli* (ATCC25722), *S. aureus* (ATCC25903), *S. sonnei* (ATCC259131), *P. aeruginosa* (ATCC27853)and *S. typhimurium* (ATCC13311).

Each medicinal plant extracted by chloroform, methanol and ethanol solvents showed a highly significant difference on inhibition zone diameters against the test of strains which ranged between 4 mm and 17 mm. The highest antibacterial activity (17 mm) was observed from chloroform leaf extract of *E. Globules* against *E. coli* (Figure 1A) and chloroform leaf extract extract of *Verbena officinalis* extract (13.6 mm) against *Shigella sonnei*, followed by methanol leaf extract of *Cordia Africana* (12.8 mm) against *E. coli*. From this study chloroform leaf extract of *Brucea antidysenterica* showed antimicrobial activity (11.0 mm) against *Pseudomonas aeruginosa*

Among all plant extracts analyzed minimum antibacterial activity was exhibited by the chloroform leaf extract of *Brucea antidysenterica* against *S. typhimurium* (4.3 mm), *Phytolacca dodecandra* against *P. aeruginosa* (4.9 mm), *Foeniculum vulgare* against *S. typhimurium* (4.7 mm) (Figure 1B) and one or more negative activity was observed from all leaf extract example all leaf extract *Foeniculum vulgare* except chloroform extract, all leaf *Phytolacca dodecandra* except chloroform extract against *P. aeruginosa* and all chloroform leaf extract *Cordia Africana* except methanol and ethanol extracts showed zero inhibition zones (Table 1).

Phytochemical screening of selected medicinal plants

Our finding confirmed that all extracted plants contain different secondary metabolites such as alkaloids, cardiac glycosides, flavonoids, saponins, tannins and phenols (Table 2).

Discussion

Evaluation of antibacterial activities of crude extracts

The highest antibacterial activity (17 mm) was observed from chloroform leaf extract of *E. globules* against *E. coli* and chloroform leaf extract of *Cordia Africana* (12.8 mm) against *E. coli* and chloroform

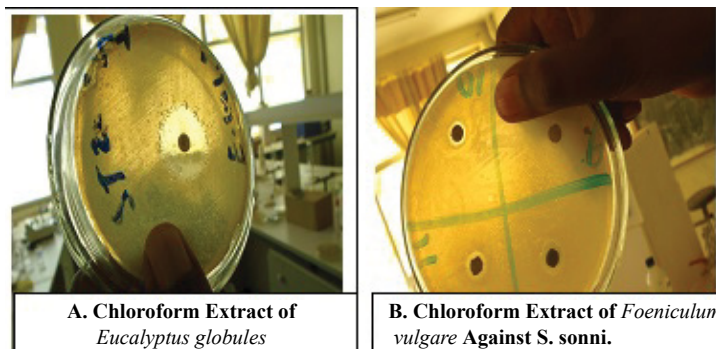


Figure 1: Zone of inhibition chloroform extract of eucalyptus globules against *E. coli* and chloroform extract of *Foeniculum vulgare* against *S. sonni*.

Scientific names	Solvent extracts	Bacterial strains				
		<i>Staphylococcus aureus</i> (ATCC25903)	<i>Salmonella typhimurium</i> (ATCC13311)	<i>Pseudomonas aeruginosa</i> (ATCC27853)	<i>Shigella sonnei</i> ATCC259131	<i>Escherichia coli</i> (ATCC 25722)
<i>Eucalyptus globules</i> Labill.	Ethanol	11.8 ± 1.04	11 ± 1.0	NA	8 ± 0.5	16.5 ± 0.5
	Methanol	10.3 ± 1.52	8.8 ± 0.28	NA	8.3 ± 1.15	16.6 ± 3.5
	Chloroform	13 ± 2.6	7.5 ± 0.86	NA	8 ± 1	17 ± 1.7
<i>Verbena officinalis</i> L.	Ethanol	10.5 ± 1.28	NA	NA	11.8 ± 1.4	12.6 ± 0.57
	Methanol	8.3 ± 0.57	NA	NA	13.1 ± 1.04	11.8 ± 0.28
	Chloroform	9 ± 2	NA	NA	13.6 ± 0.57	12.3 ± 0.57
<i>Otostegia integrifolia</i> Benth.	Ethanol	6.3 ± 1.15	NA	NA	7 ± 1	NA
	Methanol	8.6 ± 1.04	NA	NA	6.1 ± 0.28	NA
	Chloroform	9.1 ± 0.28	NA	NA	9.3 ± 1.5	NA
<i>Brucea antidysenterica</i> Swiss Chard.	Ethanol	NA	NA	NA	NA	NA
	Methanol	NA	NA	NA	NA	NA
	Chloroform	7 ± 1.0	4.3 ± 0.57	11 ± 0.0	5.5 ± 0.5	NA
<i>Phytolacca dodecandra</i> L'Herit.	Ethanol	NA	NA	NA	NA	NA
	Methanol	NA	NA	NA	NA	NA
	Chloroform	NA	NA	4.9 ± 1.36	NA	NA
<i>Cordia africana</i> Lam.	Ethanol	7.3 ± 1.15	9.1 ± 1.04	5 ± 1.0	8 ± 0.0	11.6 ± 0.57
	Methanol	5.8 ± 0.76	8.5 ± 0.5	4 ± 1.0	6.8 ± 0.76	12.8 ± 1.25
	Chloroform	NA	NA	NA	NA	NA
<i>Foeniculum vulgare</i> Miller.	Ethanol	NA	NA	NA	NA	NA
	Methanol	NA	NA	NA	NA	NA
	Chloroform	7 ± 0.0	4.7 ± 0.75	5.8 ± 0.28	5.8 ± 1.04	8.3 ± 0.57
<i>Withania somnifera</i> (L.) Dunal in DC.	Ethanol	8.1 ± 1.15	NA	NA	6.4 ± 0.85	NA
	Methanol	8.8 ± 1.4	NA	NA	5.6 ± 0.76	NA
	Chloroform	8.8 ± 1.4	NA	NA	9.8 ± 0.28	NA
Ampicilin	-	22.8 ± 0.2	21.4 ± 0.5	23 ± 0.0	24.3 ± 0.3	20.1 ± 0.45

NA-Not have Activities. Values are mean inhibition zone (mm) ± S.D of the replicates and the superscripted similar letters show absence of statistical significance (P<0.05) among the values. Statistically significance considered at p<0.05.

Table 1: Antibacterial activities of selected medicinal plants extracted by soaking method (conc. 100 mg/ml).

Tests	Name of Medicinal Plants							
	<i>Eucalyptus globules</i>	<i>Withania somnifera</i>	<i>Phytolacca dodecandra</i>	<i>Brucea antidysenterica</i>	<i>Otostegia integrifolia</i>	<i>Verbena officinalis</i>	<i>Cordia Africana</i>	<i>Foeniculum vulgare</i>
Alkaloids	+	+	+	-	+	-	-	-
Tannins	+	-	-	±	+	+	+	-
Saponins	-	-	+	-	-	+	+	+
Flavonds	+	+	+	+	+	+	-	+
Cardiac Glycosides	+	+	-	+	-	±	-	+
Phenols	+	+	+	-	-	+	-	±

Where - Indicate absence; + Indicate presence; ± Indicate slight presence/absence

Table 2: Phytochemical constituents of the selected medicinal plants.

leaf extract of *Verbena officinalis* (13.6 mm) against *S. sonnei*. Therefore, this result indicated that chloroform extracts of *E. globules*, *V. officinalis* and *W. somnifera* showed more promising activity against tested strains than other plant extracts. This suggests that the extract of this plant has a broad spectrum of antibacterial activities. The result of this study in line with the finding of previous studies reported by [23-25]. However, these antibacterial activities were observed to be significantly lower than that of ampicillin, the standard antibacterial drug used as positive control in this study. This could be due to the fact that the sample extracts used in the test were crude preparation which may not necessarily contain enough of the active chemical. Further purification and fractionation thus needed to yield more isolated bioactive compounds [26].

Otostegia integrifolia and *Withania somnifera* plant extracts showed negative effect on Gram-negative (*S. typhimurium*, *P. aeruginosa* and *E. coli*) but positive effect on Gram-positive bacterium (*S. aureus*). However, several previous findings Branter A et al. [27], Nostro et al. [28], Ojala et al. [29] and Velu and Baskaran, [24] reported that Gram-negative bacteria were susceptible to plant extracts when compared to Gram-positive bacteria. The resistance of Gram-negative bacteria towards antibacterial substances is related to lipopolysaccharides in their outer membrane [30] and [31]. This is controversial with the obtained results since most of the extracts showed prominent activity against gram-negative bacteria. Therefore, chloroform extracts of *E. globules*, *V. officinalis* and *C. Africana* which exhibited the highest inhibitory effects against gram-negative bacteria can be developed for the use in drugs industry.

Extraction of antimicrobial metabolites from the selected plant species by chloroform was observed to be more effective methods than ethanol and methanol solvents. For example, while chloroform extracts of *Foeniculum vulgare* inhibited all five tests of strains but ethanol and methanol extracts inhibited none of one strains. Thus, the efficacy of plant extracts evaluated as antimicrobial agents was dependent on the solvent of extraction. Alzoreky and Nakahara [32] reported that, chloroform was proved to be good solvents in extracting inhibitory substances from medicinal plants. In contrast, Cowan [33] found that methanol was more efficient than acetone in extracting photochemical from plant materials. In accordance with these dissimilar findings, the results of the current study revealed that the solvent type is not the only factor that should be taken in consideration during extraction of plant constituents but also the plant species as well as the test microorganism played an important role in the antimicrobial efficacy.

The test strains exhibited slight or no susceptibility to extracts of *P. dodecandra*, *B. antidisenterica* and *F. vulgare* but antibacterial activities of chloroform extract which revealed a broad spectrum activity against the test pathogenic microorganisms except *P. dodecandra* extracts. This finding is in disagreement with [34] and [35].

Preliminary phytochemical screening of the selected medicinal plants

The finding showed that *E. globules* and *Verbena officinalis* plant leave had all test compounds except Saponins and Alkaloids respectively. This correlated with the finding of previous studies that reported the presence of antimicrobial substances within the leaves of the plant as described by Saxena R et al. [36]. According to the report of Ali and Blunden [37] the inclusiveness of all these compounds has contributed to its effective activity against the test strains compared with other plants. This suggests that the extract of this plant has a broad spectrum of antibacterial activities.

According to Saxena R et al. [36], *E. globules* has essential compounds like, Alkaloids cardiac glycosides, flavonoids, tannins and phenols. To the contrary Obiorah S et al. [38] reported the absence of cardiac glycosides in the leaves of *E. globules*. Our results indicated *Withania somnifera* leaf contains Alkaloids, cardiac glycosides, flavonoids and phenols but other reports by Velu and Baskaran [24] indicated the absence of flavonoids in the leave of *Withania somnifera*. The differences in the reports of many of the study could be due to the difference in time of plant collection, climate, methods of extraction used and other factors.

Conclusions and Recommendations

Based on the results obtained in this study, all the extracts showed varying degrees of antimicrobial activity against the test strains. However, the activities varied based on the solvents used for extraction. This *in vitro* study demonstrated that some of the extracts can be as effective as modern medicine to combat the test of the strains. *E. globules*, *Otostegia integrifolia*, *Verbena officinalis*, *Cordia africana*, *Foeniculum vulgare*, *Withania somnifera*, *Phytolacca dodecandra* and *Brucea antidisenterica* have antimicrobial activity than other plants extracts to against the tested standard strains. According to results of this study, it can be concluded *E. globules*, *V. officinalis*, and *W. somnifera* leaf extract showed broad spectrum of antibacterial activity as evidenced by high zone of inhibition against the test standard strains. These results provide evidence that some of the secondary metabolites present in the studied medicinal plants confirmed as they contained multiple bio compounds which might have synergetic impact on inhibition of pathogenic microbes tested. The finding of this study also indicated that instead of using crude extracts for antimicrobial activity it could be imperative isolating bioactive compounds in its purest form and used for new drug development programs. Therefore, the authors recommend further studies to be done on isolated compounds and fractions of the plant for antimicrobial activity and toxicological screening. Moreover, *in-vivo* studies should also be conducted in order to confirm the safety and efficacy of this plant's secondary metabolites.

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Competing Interests

The authors declare that they have no competing interests.

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